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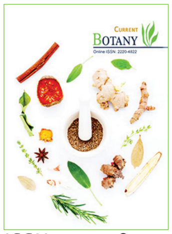

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Studies on crossability among different varieties of *Ananas comosus* (L.) Merr. (Bromeliaceae)

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ABSTRACT

Pineapple (*Ananas comosus* (L). Merr.) is a self incompatible plant. But hybridization is possible between different cultivars and varieties of pineapple. In the present study crossing experiments were carried out among different varieties of pineapple to assess the crossability among these varieties. *In vitro* pollen germination studies showed maximum pollen germination *in A. comosus* cv. MD2 in Brewbaker's medium with 25% sucrose (85.57%). In self pollinated pistil, pollen grains were germinated on the stigma and penetrated into the style, but never grew deep into the style. After cross pollination, the pollen grains germinated on the stigmatic surface, penetrated and grow deep into the style. No seed set was observed in commercial cultivars after self pollination. But, a few seeds were produced in *A. comosus* var. *bracteatus*. In cross pollination experiments, seed set was observed in all crosses. The percentage of crossability and the number of seeds per fruitlet varied among different crosses. The cross between *A. comosus* cv. Mauritius female and *A. comosus* cv. Kew male showed maximum percentage of crossability and cross between *A. comosus* cv. MD2 female and *A. comosus* cv. Mauritius male produced the maximum number of seeds per fruitlet. The percentage of seed germination showed significant variation among the seeds produced from different crosses. The seedlings transferred to pots containing potting mixture showed vigorous growth.

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INTRODUCTION

Pineapple (*Ananas comosus* (L). Merr.) is one of the economically significant fruit crops of the tropics for over a century. It belongs to the family Bromeliaceae. Propagation of pineapple is entirely done by vegetative means with the help of suckers and slips. Crowns are rarely used for propagation. Sexual reproduction is rare in nature due to self-sterility which is mainly due to the inhibition of the pollen tube growth in the upper third of the style (Majumder *et al.*, 1964). It is controlled gametophytically with a single locus with multiple alleles (Brewbaker & Gorrez, 1967). There are no reproductive barriers among botanical varieties of *Ananas comosus*. Pineapple breeders aim to produce plants with rapid growth, spineless leaves or leaves having only spines at its tips, cylindrical fruit with firm flesh, uniformly coloured with flat eyes, response to flower induction to maximize yield, increased vitamin C content, strong fruit stalk, moderate acidity and resistance to disease as well as tolerance to some herbicides. High sucrose content and pleasant aroma are also essential. So artificial pollination is preferred for hybridization. Pineapple cultivars are heterozygous and hybridization between them leads to the production of fertile seeds. Many countries have started hybridization programmes to develop high yielding varieties of pineapple with specific adaptations to their own environment, e.g. Taiwan (Fitchet, 1989), Malaysia (Chan, 1997; Wee, 1974), Phillipines, Cuba and Australia (Ram´ırez *et al.*, 1972; Cabot, 1987; Cabral *et al.*, 1993).

In India, hybridization experiments were done at Kerala Agricultural University, Vellanikkara and developed a hybrid called 'Amritha' (hybrid of cultivar 'Kew' and 'Ripley Queen'). Another hybrid variety of pineapple, 'MD2' is imported to Kerala and successfully grown in the field of Pineapple research station, Kerala Agricultural University, Vazhakkulam. The American Society for Horticultural Science called 'MD2' as 'outstanding fruit cultivar' in the year 2010. The pineapple breeding programmes and field trials of the hybrids are going on in Kerala Agriculture University, Vellanikkara. The hybrid variety 'Amritha' is the only variety released in Kerala till now. Of the four different varieties cultivated in Kerala, 'Mauritius' has high consumer preference due to its better fruit qualities. The variety proposed for cultivation in Kerala is also 'Mauritius' since a huge internal market and export potential is available for it. It has a longer shelf life, sweetness and good aroma. But, the other commercial cultivars and local varieties also have certain good qualities. The purpose of carrying out the crossability studies therefore is to prepare baseline data for the production of combinations that might have a better balance of desirable characters, compared with existing varieties. So the present study aims to analyze the crossability between different varieties cultivated in Kerala.

MATERIALS AND METHODS

For the crossability study, the four commercial cultivars of pineapple in Kerala, 'Mauritius' (ACM), 'Kew'(ACK), 'MD2'(ACMD) and 'Amritha'(ACA) and two local varieties, *Ananas comosus* var. *bracteatus*-red (ACBR) and *A. comosus* var. *bracteatus*-green (ACBG) were selected. Prior to hybridization experiments, the *in vitro* pollen germination studies of all the selected varieties were done. After that the crossing experiments were carried out.

In Vitro Pollen Germination

In vitro germination of the pollen grains was carried out in Brewbaker and Kwack's medium (Brewbaker & Kwack, 1963). This medium consists of boric acid (10 mg), potassium nitrate (10 mg), magnesium nitrate (20 mg) and calcium nitrate (30 mg) in 100 mL distilled water. Along with these standard ingredients, 5-50% sucrose was supplemented separately to the medium. Pollen grains collected from mature buds, just before anther dehiscence, were dusted on a drop of culture medium placed on a clean glass slide and incubated in a germination chamber. The chamber used in the study was a pair of petridishes lined with moist cotton. For each concentration of sucrose five slides were prepared. These slides were kept undisturbed at room temperature. After two hours, the total number of pollen grains and germinated pollen grains from 20 fields of each slide were estimated, recorded and percentage of germination was calculated.

Crossability Study

Crossability studies were carried out in the plants cultivated in the experimental field at the Department of Botany, University of Kerala, Kariavattom, Thiruvananthapuram. The different cultivars are planted in different sites in the field to avoid cross pollination. Flowering was induced in 35-40 leaved stage by hormonal application with Ethephone (0.025 mL/L) in combination with 2% urea and 0.04% of sodium carbonate. Each plant was applied 50 mL of the hormone. After 45 days, inflorescence emerged from the plants and they were covered with pollination bags. Self and cross pollinations were done in these plants from October 2014 to February 2015. Patterns of crossing among the varieties were shown in Table 1. Thirty cross pollinations (including reciprocal crosses) and six self pollinations were done. Daily, in the early morning i.e. around 6 AM the anthers from male parents were collected in a petri dish using forceps and were used either to self pollinate or cross pollinate manually in bloomed flowers. The pollen grains from the male plant were dusted over the stigma of the female plant and covered using pollination bags to avoid the interference of other pollen.

Table 1: Pattern of crossing among different varieties of *Ananas comosus*

♂ \ ♀	ACMR	ACK	ACMD	ACA	ACBR	ACBG
ACMR	-	✓	✓	✓	✓	✓
ACK	✓	-	✓	✓	✓	✓
ACMD	✓	✓	-	✓	✓	✓
ACA	✓	✓	✓	-	✓	✓
ACBR	✓	✓	✓	✓	-	✓
ACBG	✓	✓	✓	✓	✓	-

In Vivo Pollen Germination

In vivo pollen germination studies were carried out using Aniline blue Fluorescence method (Martin, 1959). Ten hours after pollination, self and cross pollinated pistils were collected and fixed in Carnoy's fluid for about 24 hrs and stored in 70% ethanol for further studies. The fixed pistils were transferred to 4 N NaOH for 6 hours to make the tissue soft. Then the pistils were washed thoroughly with distilled water and stained with 0.1% aniline blue in 0.1 M K_2HPO_4 solution for overnight. Stained pistils were mounted in a drop of glycerine. Apply gentle pressure on the cover glass to achieve the required degree of spreading of the tissue. Pollen germination and pollen tube growth after self and cross pollinations were assessed using a confocal microscope (Leica TCS SP 8).

Study on Seed Set and Seed Germination

Pollinated flowers were counted and labelled. The number of flowers crossed varied from one plant to another depending on the availability of bloomed flowers at that time. At maturity, fruits were harvested, weighed, the number of fruitlets showing seedset, seeds contained in each fruitlet and weight of seeds were scored. The percentage of crossability was calculated. Seeds produced were then allowed to germinate in petri dish lined with moist cotton. These seeds were found germinated after 25-40 days. Percentage of seed germination was calculated after 40 days. The seedlings were then transferred to small pots filled with sand for one month and were subsequently transplanted to medium sized pots containing mixture of soil, manure and sand (1:1:1) for six months. After that, the seedlings were transferred to large pots containing the same potting mixture until the plants produced flowers. Collected data were subjected to one way ANOVA using SPSS version 16.

RESULTS AND DISCUSSION

In Vitro Pollen Germination

In vitro test was conducted to assess the percentage of viable pollen grains. Pollen germination commenced in all cultivars after two hours in medium supplemented with different concentrations of sucrose. The percentage of pollen germination in different varieties of pineapple for different concentrations of sucrose are depicted in Figure 1. In cultivar 'Mauritius' and A. *comosus* var. *bracteatus*-red, maximum germination of 65.69% and 80.83% were noticed respectively in medium with 20% sucrose. The germination percentage was found to be maximum in medium with 25% sucrose in cultivar 'Kew' (64.5%), 'MD2' (85.57%) and 'Amritha' (67.26%). In vitro pollen germination technique is widely used to test the viability of pollen grain. A carbohydrate source is essential for pollen germination. Sucrose is the most commonly used carbohydrate source in culture medium which serves the maintenance of osmotic pressure of the medium and carbon source for metabolism (Shivanna & Johri, 1989). The optimum sugar concentration necessary for pollen germination varies considerably with species (Johri & Vasil, 1961). Boron and Calcium also play a great role in pollen germination (Brewbaker & Kwack, 1963; Steer & Steer, 1989; Rane *et al.*, 2023). Brewbaker and Kwack's medium containing sucrose and minerals is widely used for in vitro pollen germination. In the present study, the different varieties of pineapple showed maximum pollen germination in Brewbaker and Kwack's medium supplemented with 20 or 25% sucrose. In pineapple, *in vitro* pollen germination was reported in certain cultivars (Wee & Rao, 1979). Since the percentage of pollen germination is higher in different varieties of pineapple, it is not a reason for lack of seed set in this plant after self pollination. In many plant species, in vitro pollen germination showed a positive correlation with fruit and seed set (Janssen & Hermsen, 1976). A negative correlation was observed between pollen germination and seed set after self fertilization in different varieties of A. *comosus*. Due to heterozygosity, crossing is possible between different varieties of pineapple. So the high percentage of pollen germination may be helpful for seed set after cross pollination.

Crossability Studies

Thirty cross pollination and six self pollination experiments were carried out among different varieties of pineapple.

In Vivo Pollen Germination Studies After Self and Cross Pollination

After self pollination, the pistils stained with aniline blue showed that the pollen grains are adhered and germinated on the stigmatic surface (Figure 2a). The pollen grains penetrated into the style, but never grow deep into the style. In pineapple, the self incompatibility is generally associated with the inhibition of pollen tube growth in the style. The germ cells produced in A. *comosus* are functional but they show self sterility due to gametophytic incompatibility. In gametophytic self incompatibility, self as well as non-self pollens are initially germinated on the stigma surface. But only the pollen tube of non-self pollen can enter the ovary and the pollen tube growth of self pollen is prevented in the style (Chakraborty *et al.*, 2023). This gametophytic self incompatibility is controlled by a single 'S' locus with multiple alleles (Brewbaker & Gorrez, 1967). The inhibition of pollen tube growth mainly takes place in the upper third of the style commonly in the stigmatic lobes (Majumder *et al.*, 1964). This has been confirmed in the present work. In Singapur Spanish cultivar also the pollen tube growth was limited in four hours after selfing (Wee & Rao, 1979). After cross pollination, large number of pollen grains adhered and germinated on the stigmatic surface (Figure 2b). The compatible pollen tubes penetrated into the stigma and grew down into the style (Figures 2c & d). When the pollen grains of cultivar 'Mauritius' placed on the stigmatic surface of Mesmerah cultivar, fertilization was observed within two hours and the rate of tube growth was high (Wee & Rao, 1979).

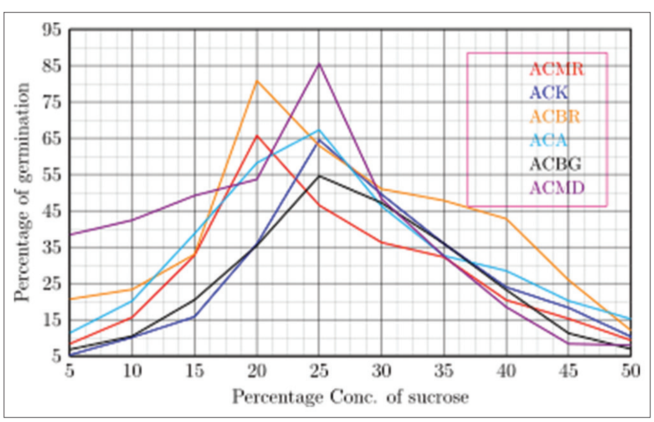

Figure 1: *In vitro* pollen germination of different varieties of *A. comosus*

Outcome of Self and Cross Pollination Experiments

The result of crosses pertaining to crossability and percentage of seed germination are presented in Table 2. In self pollinations, no seed set was observed in all four commercial cultivars studied. It may be due to self incompatibility in pineapple. Strong self incompatibility was already reported in different cultivars of pineapple (Coppens d' Eeckenbrugge *et al.*, 1993). But the local variety, A. *comosus* var. *bracteatus* produced very few seeds from some flowers. It is due to pseudo self incompatibilty. Seed formation is more frequent among the wild pineapple (Collins, 1960). Self incompatibility and pseudo self incompatibility were reported in certain varieties of A. *comosus* (Duval & Coppens d'Eeckenbrugge, 1993). All commercial cultivars of pineapple belong to A. *comosus* var. *comosus*. The self incompatibility is stronger in this variety than other varieties of A. *comosus*.

In pollination experiments, seed set was noticed in all crosses. But, the percent crossability varied among different crosses. Maximum percentage of crossability (98%) was observed in cross between A. *comosus* cv. Mauritius (♀) and A. *comosus* cv. Kew (♂). Good percentage of crossability was observed in its reciprocal cross also. Cultivar 'Mauritius' belongs to 'queen' group of pineapple and cultivar 'Kew' belongs to 'Cayenne' group. The dendrograms from morphological and molecular characterization studies grouped the accessions of cultivar 'Mauritius' and cultivar 'Kew' in two distinct clusters (Nisha, 2019). It also proves the genetic variability among these cultivars. They are genetically diverse groups. Crossability in pineapple is high in cross between such genetically diverse groups. In eleven crosses the percentage of crossability was above 90%, in nine crosses it was between 80% and 90%, in seven crosses the percentage of crossability is between 50 and 80 and

Table 2: Results of hybridization studies between different varieties of *A. comosus*

S. No.	Crosses	No. of flowers pollinated	No. of flowers showing seed set	Crossability percentage	No. of seeds/ fruitlet	Weight of ten seeds (mg)	Percentage of seed germination
1	ACK(♀) x ACM(♂)	40	36	90	2.9±0.27	82.08±0.43	60±2.58
2	ACMD(♀) × ACM(♂)	35	34	97	8.5±0.42	59.56±0.82	34±2.21
3	ACA(♀) × ACM(♂)	20	14	70	1.3±0.26	66.03±0.56	65±1.49
4	ACBG(♀) × ACM(♂)	35	30	85.71	1.7±0.21	96.89±1.51	58±2.49
5	ACBR(♀) × ACM(♂)	25	21	84	1.6±0.16	94.47±1.32	60±2.10
6	ACM(♀) × ACK(♂)	50	49	98	3.7±0.3	87.35±0.64	50±2.58
7	ACA(♀) × ACK(♂)	28	15	53.57	1.1±0.23	79.20±0.41	17±1.52
8	ACMD(♀) × ACK (♂)	28	26	92.86	7.7±0.74	53.83±0.78	25±1.66
9	ACBG(♀) × ACK(♂)	35	32	91.43	2.5±0.16	86.56±1.33	24±2.21
10	ACBR(♀) × ACK(♂)	30	27	90	3.2±0.24	92.50±0.47	44±2.21
11	ACM(♀)×ACMD(♂)	46	44	95.65	5.8±0.84	56.97±0.38	51±2.33
12	ACK(♀) × ACMD(♂)	42	38	90.48	7.5±0.31	81.23±0.38	41±2.33
13	ACA(♀) × ACMD(♂)	16	7	43.75	3.7±0.3	81.23±0.45	28±1.28
14	ACBG(♀)×ACMD(♂)	26	22	84.62	1.7±0.15	79.74±1.75	58±3.88
15	ACBR(♀)× ACMD(♂)	22	18	81.82	2.4±0.16	93.56±0.53	52±0.98
16	ACM(♀) × ACA(♂)	16	8	50	0.8±0.24	54.16±0.39	16±1.63
17	ACK(♀) × ACA(♂)	15	13	86.67	2.1±0.27	80.77±0.48	75±1.66
18	ACMD(♀) x ACA(♂)	23	21	91.3	6.1±0.23	58.45±0.26	32±1.56
19	ACBG(♀)× ACA(♂)	24	19	79.16	0.9±0.23	96.64±0.57	65±1.66
20	ACBR(♀) xACA(♂)	22	18	81.82	1.4±0.16	93.38±0.57	52±1.80
21	ACM(♀)× ACBR(♂)	42	38	90.48	3.9±0.31	91.89±0.93	95±1.66
22	ACK(♀)× ACBR(♂)	18	16	88.89	1.6±0.16	80.94±0.43	64±2.14
23	ACMD(♀)× ACBR(♂)	15	10	66.67	1.5±0.16	54.2±0.32	23±2.13
24	ACA(♀) xACBR(♂)	28	25	89.29	3.3±0.27	78.46±0.60	34±1.86
25	ACBG(♀)× ACBR(♂)	18	8	44.44	0.8±0.2	88.9±0.28	20±0.95
26	ACM(♀)× ACBG(♂)	22	16	72.72	0.6±0.22	87.24±0.18	48±2
27	ACK(♀) × ACBG(♂)	13	11	84.62	2.7±0.27	82.1±0.42	52±1.25
28	ACMD(♀)× CBG(♂)	32	30	93.75	4.7±0.33	58.83±0.96	25±1.66
29	ACA(♀) × ACBG(♂)	30	22	73.33	2.0±0.21	77.58±0.62	44±1.5
30	ACBR(♀)× ACBG(♂)	24	10	41.67	0.5±0.17	90.45±0.36	21±1.5

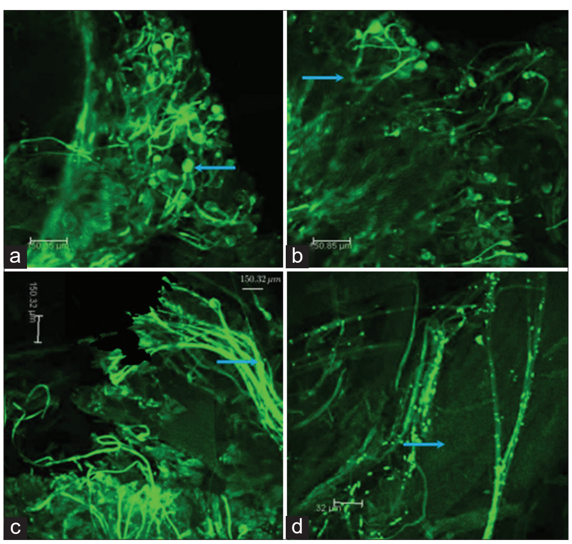

Figure 2: *In vivo* pollen germination a) Pollen germination on the stigmatic surface after cross pollination, b) Pollen germination on the stigmatic surface after self pollination, c) Pollen tubes penetrated into the style and d) Pollen tubes that grow deep into the style

in three crosses it is below 50%. Percentage of crossability was very low in cross between A. *comosus* var. *bracteatus*-red and A. *comosus* var. *bracteatus*-green.

The number of seeds per fruitlet among different crosses varies significantly. Maximum number of seeds per fruitlet (8.5) was observed in cross between A. *comosus* cv. MD2 female and A. *comosus* cv. Mauritius male (Figures 3a & b). This is due to the least genetic similarity between cultivar 'MD2' and cultivar 'Mauritius'. The lowest number of seeds per fruitlet was observed in cross between A. *comosus* var. *bracteatus*-red (♀) and A. *comosus* var. *bracteatus*-green (♂). The percentage of crossability was high in the majority of crosses where cultivar 'MD2' was one parent except in cross between cultivar 'MD2' and cultivar 'Amritha'. 'MD2' is a hybrid variety. So it is highly heterozygous. This heterozygosity increases the rate of crossability. But the molecular characterization studies of these cultivars showed that the cultivar 'MD2' has more genetic similarity with cultivar 'Amritha' than other cultivars (Nisha, 2019). This genetical similarity leads to the low rate of crossability between these varieties. The percentage of crossability and number of seeds per fruitlet were less in the same cross ie, A. *comosus* var. *bracteatus*-red (♀) ×A. *comosus* var. *bracteatus*-green (♂). In its reciprocal cross also the result is almost same. In this cross both parents are of the same variety. They showed only some colour difference in certain morphological traits. In molecular characterization using ISSR markers, the banding patterns are almost similar and clustered together in the dendrogram (Nisha, 2019). The seed set in this cross is due to pseudo self incompatibility. But when A. *comosus* var. *bracteatus* was crossed with the commercial cultivars of A. *comosus* var. *comosus*, good percentage of crossability was noticed in all these crosses. This is due to the cross compatibility between these varieties.

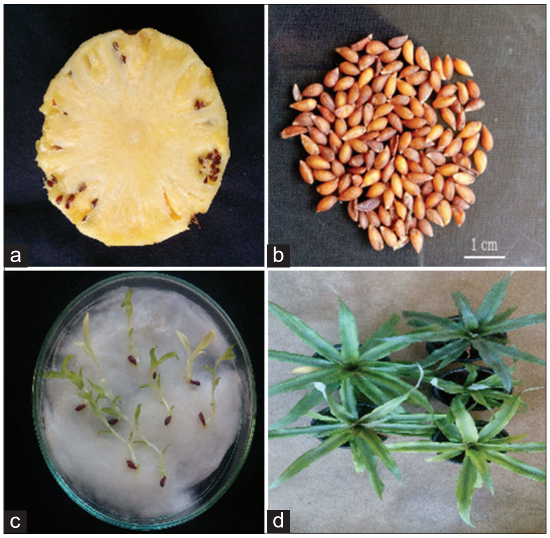

Figure 3: Seed set, Seed germination and hybrid plants. a) seed set in fruit, b) Seeds, c) Seed germination and d) Six months old hybrid plants

Seeds were separated from the fruitlets and the weight of ten seeds was scored. Maximum seed weight was recorded in seeds obtained (96.89 mg/10 seeds) from cross between A. *comosus* var. *bracteatus*-green (♀) and A. *comosus* cv. Mauritius (♂) and lowest was recorded in seeds obtained from cross between A. *comosus* cv. MD2 (♀) and A. *comosus* cv. Kew (♂) (53.83 mg/10 seeds). The percentage of seed germination was maximum (95%) in A. *comosus* cv. Mauritius (♀) × A. *comosus* var. *bracteatus*-red (♂) (Figure 3c). It was less (16%) in A. *comosus* cv. Mauritius (♀) × A. *comosus* cv. Amritha (♂). The seedlings, transferred to small pots (Figure 3d) for six months and then to large pots containing potting mixture, showed vigorous growth.

CONCLUSION

In the present study, all varieties showed a good percentage of *invitro* pollen germination. So the pollen germination capacity is not a reason for self in-compatibility. This high percentage of pollen germination had a positive effect on the crossability between different cultivars and varieties. After self and cross pollination experiments, the aniline blue stained pistils showed a good percentage of pollen germination on the stigmatic surface. But in self pollinated pistils, the pollen tubes penetrate the style but do not grow deep into it. In cross pollinated pistils it was observed that the pollen tubes penetrated deep into the style. In crossability studies among different cultivars and varieties, seed set was noticed in all crosses. But the percentage crossability, number of seeds per fruitlet, seed weight and percentage of seed germination vary among different crosses. So it was concluded that crossing is possible between all varieties of pineapple. So better varieties can be produced by hybridization between different varieties. Further field evaluation of the hybrids is necessary for the selection of hybrids with better qualities.

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